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Brief Report

Targeting β-catenin dependent Wnt signaling via peptidomimetic inhibitors in murine chondrocytes and OA cartilage

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SUMMARY

Objective: The canonical Wnt signaling pathway has been shown to be involved in regulating chondrocyte hypertrophic differentiation during Osteoarthritis (OA). The aim of this study was to test the therapeutic potential of two stapled peptide canonical Wnt inhibitors – SAH-Bcl9 and StAx-35R – in preventing Wnt induced cartilage changes in OA.
Methods: Primary neonatal murine chondrocytes and cartilage explants from OA patients undergoing total joint replacement for knee OA, were used for microscopy to determine matrix and cell penetrating capacity of fluorescein isothiocyanate FITC-tagged SAH-Bcl9 and StAx-35R peptides. T cell factor/lymphoid enhancer-binding factor (TCF/LEF) reporter assays were used to monitor the inhibition of Wnt3a induced β-catenin signaling by each peptide. Changes in chondrocyte phenotypic marker gene expression were analyzed by qRT PCR.
Results: Both peptides localized intercellular in primary murine chondrocytes and cartilage explants. They inhibited Wnt3a induced TCF/LEF promoter activity in primary murine chondrocytes. Both inhibitors did not rescue Wnt3a altered expression of chondrocyte phenotypic genes (Sox9, Col2a1, Acan) and hypertrophy marker gene (Col10a1) at high doses (100 ng/ml). Upon application of 10 ng/ml Wnt3a, StAx-35R partially reversed the Wnt effect on Sox9 and Col2a1 gene expression. Both peptides, however, reversed the downregulation of SOX9 and aggrecan (ACAN), and decrease of COL10A1 gene expression in preserved human OA cartilage explants.
Conclusion: These data indicate that blockade of canonical Wnt signaling might be a therapeutic strategy to treat early OA cases and protect further cartilage degradation by preventing chondrocyte hypertrophic differentiation.

Introduction

Osteoarthritis (OA) is characterized by progressive destruction of articular cartilage. One central pathologic feature is the phenotypic shift of chondrocytes from resting towards hypertrophic. Among others, this shift is triggered by activation of canonical Wnt/β-catenin signaling. Enhanced expression of Wnt signaling components has been shown in various OA systems[1,2]. Activation of the canonical pathway alters the characteristic expression pattern of chondrocytes, resulting in decreased type II collagen (COL2A1), aggrecan (ACAN), SOX9, and in increased type X collagen (COL10A1) expression, and therewith promoting cell de-differentiation[1,3]. Conclusively, therapeutic inhibition of canonical Wnt/β-catenin signaling to maintain chondrocyte phenotypic stability and therewith cartilage homeostasis is desirable for OA treatment.

In absence of Wnt signaling, cytosolic β-catenin is degraded. Upon Wnt ligand binding to its receptors Frizzled (Fz) and low-density lipoprotein-related receptor protein 5 and 6 (LRP5/6) the β-catenin gets stabilized, accumulates in the cytoplasma and translocates into the nucleus. Here, β-catenin binds T cell factor/lymphoid enhancer-binding factor (TCF/LEF) and mobilizes other co-activators such as the B-cell lymphoma protein (Bcl9) to induce gene transcription[4].

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Distinct regulation of the canonical Wnt signaling is required to prevent the development of various diseases including OA and cancer. Therefore, siRNAs, antibodies and small molecule inhibitors are under continuous investigation to interfere with Wnt signal transduction[5,6]. Two small molecule inhibitors, the stapled peptides StAx-35R (stapled β-catenin binding domain of Axin) and SAH-Bcl9 (stapled peptide derived from the Bcl9 homology domain-2), have been established to inhibit β-catenin transcriptional activity and thereby canonical Wnt signaling mediated tumor growth in cancer[7,8]. Both inhibitors of canonical Wnt signaling mimic binding domains that directly interact with β-catenin and disrupt β-catenin transcriptional function. We tested SAH-Bcl9 and StAx-35R for their ability to prevent canonical Wnt signaling induced hypertrophic differentiation of chondrocytes in OA.

Methods

Chondrocyte isolation and cartilage explants

Murine chondrocytes of knee cartilage were isolated from 5 to 8 days old neonatal C57Bl/6 mice as described[9]. All experiments were performed using freshly isolated chondrocytes, which were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate and penicillin (10.000 U/ml)/streptomycin (10 mg/ml).

Human OA articular cartilage was obtained from patients undergoing joint replacement for knee OA after obtained consent (ethics number: 23/16). Full thickness samples were dissected from preserved and OA areas of articular cartilage of OA patients by macroscopic distinction between loaded (OA) and non-loaded (preserved) cartilage areas. Explants were kept in culture medium (see above) without further treatment for 48 h until application of Wnt inhibitors.

Peptide synthesis and characterization

Peptides were synthesized as recently reported and described in detail in Supplemental Material. Fluorescein isothiocyanate FITC-tagged inhibitors were applied to cells, preserved or OA cartilage explants for microscopic analysis of cell penetration. Acetylated (Ac-) SAH-Bcl9 and StAx-35R were used for *in vitro* cell culture and explant experiments for subsequent gene expression analysis. As Ac-SAH-Bcl9 and Ac-StAx-35R were solved in dimethylsulfoxide (DMSO), DMSO has been introduced as vehicle control in the respective experiments.

RNA extraction, cDNA synthesis, real-time RT-PCR

Total RNA was extracted from cells and cartilage explants using TRIzol reagent (Invitrogen). 1 ng of total RNA from each sample was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using oligo dT primers. Quantitative polymerase chain reaction (PCR) was performed with SYBR Green I (SG) asymmetrical cyanine dye (SYBR) green using Applied Biosystems™ PRISM 7900HT (Thermo Scientific). Primer sequences are listed in Suppl. Tables 2 and 3. Absolute quantification was carried out using standard curves. Target gene expression was normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH).

Transfection and luciferase reporter assay

Subconfluent chondrocytes were co-transfected with Super 8XTOPFlash TCF/LEF-firefly luciferase reporter vector and cytomegalovirus CMV-Renilla luciferase vector (ratio 1:1, 0.75 μg pDNA/well) using jetPRIME (Polypus). Transfection efficiency was determined by microscopic counting of green fluorescent protein (GFP) positive cells in a separate well as transfection control (approximately 30% in each experiment). 24 h after transfection, cells were pre-incubated with Ac-SAH-Bcl9, Ac-StAx-35R or vehicle for 1 h before treatment for 24 h with 100 ng/ml rmWnt3a (R&D Systems). Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) and GloMax 20/20 Luminometer (Promega), Infinite® 200 Pro (Tecan) spectrometer. Firefly luciferase activity was normalized to Renilla luciferase activity and to the vehicle control.

Microscopy of FITC-tagged peptides

To investigate intracellular penetration of peptides, murine chondrocytes were incubated with 10 μM FITC-SAH-Bcl9 or FITC-StAx-35R for 24 h. Afterwards the cells were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde. Localization was determined by confocal fluorescence microscopy (Zeiss LSM 700 confocal microscope). 4′,6-Diamidin-2-phenylindol (DAPI) was used for nuclear staining. Preserved or OA human cartilage explants were treated with 10 μM FITC-SAH-Bcl9/FITC-StAx-35R for 24 h. Afterwards the explants were washed, fixed with Tissue Tek® and directly frozen in liquid nitrogen. Sections were fixed in acetone-methanol, subsequently washed and stained with Roti®-Mount FluorCare DAPI (Roth).

β-Catenin immunofluorescence

Paraffin sections from OA cartilage were rehydrated. For antigen retrieval sections were pretreated with 0.02% HCl and then incubated with pepsin (0.25 mg/ml in 0.02% HCl) for 45 min at 37°C. Free epitopes were blocked with 4% bovine serum albumin (BSA) in PBS for 1 h at RT. Cartilage sections were stained with primary antibody against β-catenin (1:70, Cell signaling #9562). Alexa Fluor® 555 (Thermo Scientific) was applied as secondary antibody. Sections were fixed with Roti®-Mount FluorCare DAPI.

Statistics

All data were presented as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) following a Least Significant Difference-Bonferroni Test (LSD) test as *post-hoc* test in case of a significant ANOVA result. Data analyses were performed using PASW Statistics 24 (SPSS, Chicago, IL, USA). Statistical significance was determined at level of $P < 0.05$. $^{*}P < 0.05$; $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

Results

Wnt inhibitors reduced Wnt3a induced β-catenin reporter activity and modified chondrocyte marker gene expression in primary murine chondrocytes

Confocal fluorescence microscopy confirmed intracellular localization of both, FITC-SAH-Bcl9 and -StAx-35R, peptides [Fig. 1(A)]. To evaluate the dose-dependent inhibitory capacity of 100 nM, 5 μM, 10 μM and 15 μM Ac-SAH-Bcl9 or -StAx-35R, we activated the TOPFlash canonical Wnt reporter in primary chondrocytes using 100 ng/ml Wnt3a [Fig. 1(B)]. Activation of this TCF/LEF reporter assay was confirmed using 10 and 100 ng/ml Wnt3a (Suppl. Fig. 1). Treatment with 10 μM inhibitor upon stimulation with 100 ng/ml Wnt3a resulted in a significant reduction of TCF/LEF promoter activity ($F = 3.94$, $P < 0.05$) of 57.8% for Ac-SAH-Bcl9 (mean ± SEM: 0.42 ± 0.24, $P = 0.03$) and 74.2% for Ac-StAx-35R

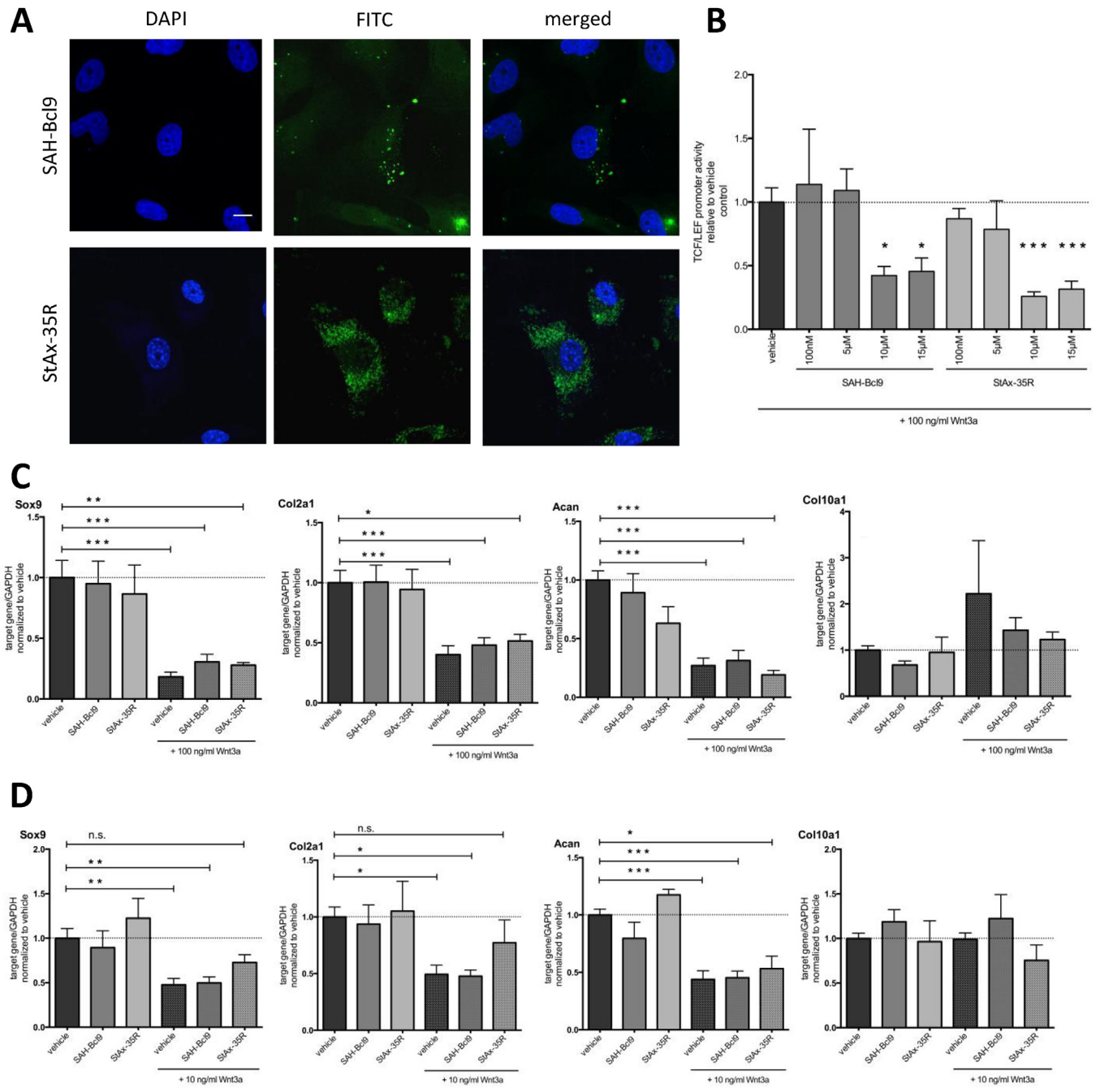

Fig. 1. Wnt inhibitors reduced Wnt3a induced β-catenin reporter activity and modified chondrocyte marker gene expression in primary murine chondrocytes. Confocal microscopy confirmed cytosolic localization of FITC-tagged SAH-Bcl9 and StAx-35R peptides (maximum intensity of z stacks, scale bar = 10 µM, nucleus staining: DAPI (blue), **A**). In comparison to vehicle and Wnt3a (100 ng/ml) treated chondrocytes, application of Ac-SAH-Bcl9 or Ac-StAx-35R instead of the vehicle resulted in a dose-dependent inhibition with 10 µM as maximum inhibitory concentration for both peptides ($n = 5$ independent experiments, normalized to vehicle + 100 ng/ml Wnt3a, ANOVA: $F = 3.94$, $P \leq 0.001$, **B**). Treatment with Ac-SAH-Bcl9 did not rescue Wnt3a modulated gene expression – independently of the applied Wnt3a concentration (100 ng/ml $n = 4$, **C**; 10 ng/ml $n = 3$, **D**). Ac-StAx-35R treatment reversed Wnt3a down-regulated Sox9 and Col2a1 gene expression at the lower Wnt3a concentration (**D**).

(0.26 ± 0.16, $P \leq 0.001$), which was not further reduced with the higher inhibitor concentration of 15 µM (SAH-Bcl9 0.45 ± 0.24, $P = 0.04$; StAx-35R 0.32 ± 0.15, $P \leq 0.001$). Hence, we applied 10 µM inhibitor for all following experiments.

As both low (10 ng/ml) and high (100 ng/ml) dosages of Wnt3a were able to induce β-catenin reporter activity, both concentrations were tested regarding their effect on chondrocyte phenotypic marker gene expression. Wnt3a mediated stimulation of canonical Wnt signaling or constitutively active β-catenin resulted in increased expression of chondrocyte hypertrophy genes (e.g., Col10a1) and down-regulation of phenotypic genes (e.g., Sox9, Col2a1, Acan)[1,3,10]. We also found that treatment with 100 ng/ml Wnt3a resulted in a down-regulation of Sox9 (vehicle vs vehicle + 100 ng/ml Wnt3a: 1.0 ± 0.14 vs 0.18 ± 0.17, $P \leq 0.001$), Acan (1.0 ± 0.10 vs 0.27 ± 0.12, $P = 0.0001$) and Col2a1 (1.0 ± 0.09 vs 0.40 ± 0.12, $P \leq 0.001$) [Fig. 1(C)]. Col10a1 gene expression was upregulated upon treatment with 100 ng/ml Wnt3a (Suppl. Fig. 2, vehicle vs 100 ng/ml Wnt3a: 1.0 ± 0.091 vs

2.407 ± 0.794, $P = 0.043$). Although chondrocytes were incubated with Ac-SAH-Bcl9 or Ac-StAx-35R prior to Wnt3a stimulation, inhibitors were not able to rescue 100 ng/ml Wnt3a modified gene expression.

With the lower dosage of 10 ng/ml Wnt3a a similar down-regulation of Sox9 (vehicle vs vehicle + 100 ng/ml Wnt3a: 1.0 ± 0.11 vs 0.48 ± 0.12, $P = 0.003$), Acan (1.0 ± 0.09 vs 0.44 ± 0.12, $P \leq 0.001$) and Col2a1 (1.0 ± 0.11 vs 0.52 ± 0.14, $P = 0.012$) expression was

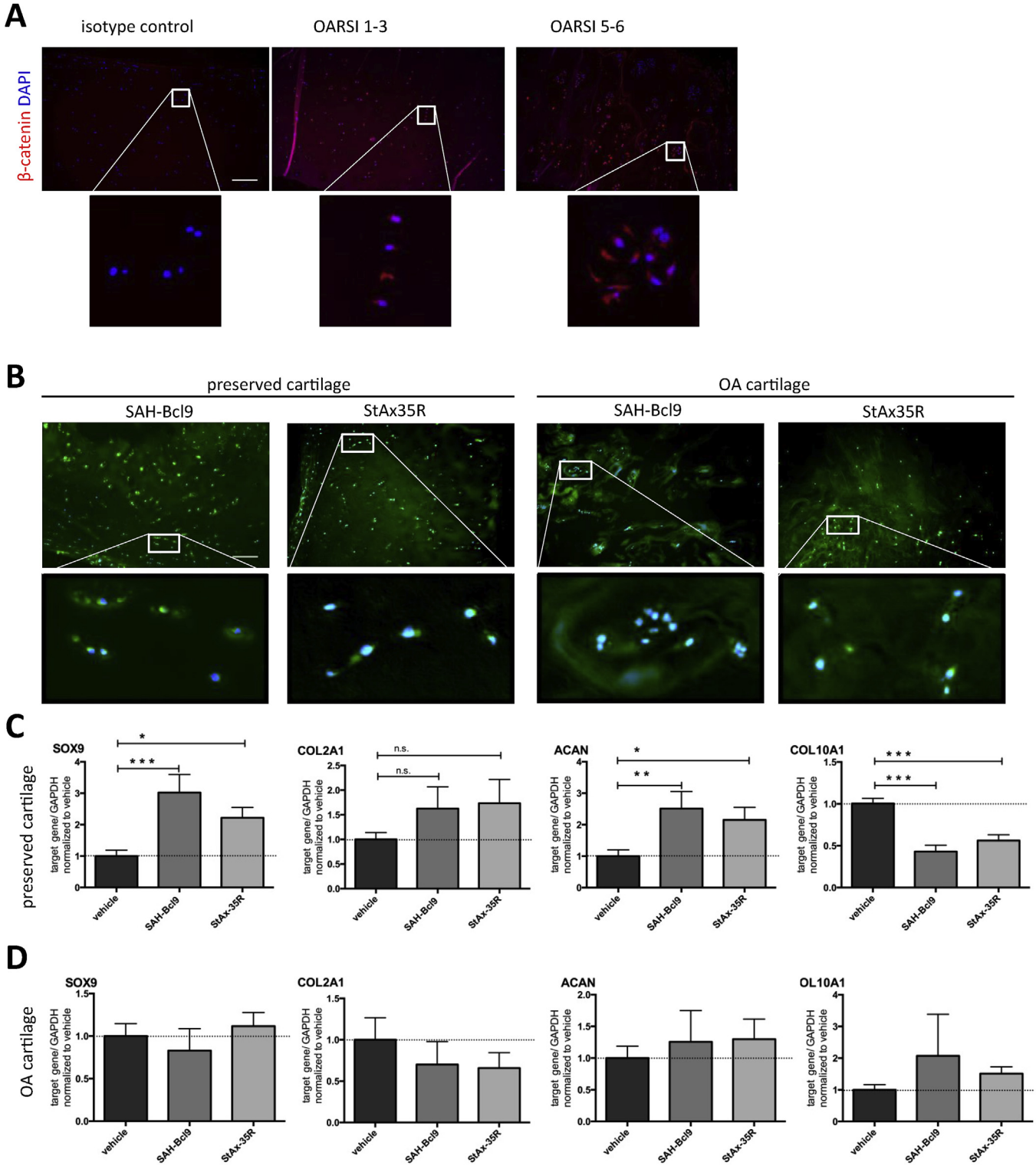

Fig. 2. Wnt inhibitors protected the chondrocyte phenotype in human preserved OA cartilage. Increasing β-catenin staining was observed with increasing OA grade (β-catenin – Alexa 555 (red), nucleus staining: DAPI (blue), scale bar = 200 μm, **A**). FITC-tagged SAH-Bcl9 and StAx-35R can penetrate into preserved and OA cartilage without any pre-treatment as shown by microscopy of cartilage explants (scale bar = 200 μm, **B**). qRT-PCR analysis revealed significantly increased SOX9, ACAN and decreased COL10A1 gene expression in preserved cartilage ($n = 6$ patients, **C**) upon 24 h treatment with β-catenin/Wnt signaling inhibitors, which was not present in highly damaged OA cartilage samples ($n = 3$ patients, **D**).

observed. Col10a1 gene expression upon stimulation with 10 ng/ml Wnt3a remained unchanged [Fig. 1(D); Suppl. Fig. 2]. Treatment with Ac-SAH-Bcl9 did not reverse the Wnt-induced effect on anabolic marker gene expression. Interestingly, treatment with Ac-StAx-35R partially inhibited the effect of 10 ng/ml Wnt3a on Sox9 (vehicle vs Wnt3a + StAx-35R: 1.0 ± 0.11 vs 0.73 ± 0.19, $P = 0.23$) and Col2a1 (vehicle vs Wnt3a + StAx-35R: 1.0 ± 0.11 vs 0.77 ± 0.19, $P = 0.32$) gene expression. This finding gave rise to the assumption that the inhibitors might be able to inhibit the phenotypic shift of chondrocytes in OA. Therefore we used an *ex vivo* cartilage culture model of OA, with disease dependent activation of canonical Wnt signaling.

Wnt inhibitors protected the chondrocyte phenotype in human preserved OA cartilage

It has been shown that β-catenin gets stabilized in OA cartilage and increases upon disease progression[1,10]. To test whether β-catenin as prominent effector of canonical Wnt signaling is present in our experimental setting, we stained for this protein. We observed an increased β-catenin staining with increasing OA grade, which corroborates the activation of canonical Wnt signaling in human OA cartilage [Fig. 2(A)].

Thus, we used preserved and OA lesion cartilage explants isolated from OA knees and incubated these with FITC-tagged peptides. Again, fluorescence microscopy proved the ability of both − SAH-Bcl9 and StAx-35R − to penetrate into preserved as well as OA cartilage [Fig. 2(B)]. Intriguingly, incubation with 10 μM Ac-SAH-Bcl9 or Ac-StAx-25R for 24 h resulted in significant up-regulation of SOX9 (vehicle vs Ac-SAH-Bcl9: 1.0 ± 0.42 vs 3.02 ± 0.40, $P = 0.001$; vehicle vs Ac-StAx-35R: 1.0 ± 0.42 vs 2.22 ± 0.38, $P = 0.037$) and ACAN (vehicle vs Ac-SAH-Bcl9: 1.0 ± 0.38 vs 2.51 ± 0.39, $P = 0.009$; vehicle vs Ac-StAx-35R: 1.0 ± 0.38 vs 2.15 ± 0.41, $P = 0.048$) and down-regulation of COL10A1 (vehicle vs Ac-SAH-Bcl9: 1.0 ± 0.08 vs 0.43 ± 0.08, $P = 0.001$; vehicle vs Ac-StAx-35R: 1.0 ± 0.08 vs 0.56 ± 0.06, $P = 0.01$) gene expression in preserved cartilage [Fig. 2(C)].However, in OA cartilage this effect was not observed [Fig. 2(D)].

Discussion

As treatment of OA is frequently symptomatic, using e.g., non-steroidal anti-inflammatory drugs (NSAIDs) or intraarticular steroids, and is mostly resulting at end-stage in total joint replacements, a treatment to prevent chondrocyte differentiation during OA is desirable[11]. Consistently with other studies (e.g.[1,2]), we have shown that canonical Wnt signaling is activated with increasing OA disease severity [Fig. 2(A)], assuming that blockade of this signaling cascade with small inhibitors prevents disease progression.

We tested SAH-Bcl9 and StAx-35R for their therapeutic potential in OA as they are already established for cancer treatment. Both inhibitors penetrated into primary chondrocytes [Fig. 1(A)] and cartilage explants [Fig. 2(B)]. They showed a dose-dependent inhibition of Wnt3a induced β-catenin reporter activity, exhibiting a half maximal inhibition at 10 μM [Fig. 1(B)].

However, only the use of 10 ng/ml Wnt3a resulted in a chondroprotective effect upon StAx-35R treatment, with regard to Sox9 and Col2a1 expression [Fig. 1(D)]. The minor inhibitory effect on Wnt3a modulated gene expression in isolated chondrocytes might be attributed to the complexity of Wnt signaling. It has been shown that inhibition or over-activation of canonical Wnt signaling results in OA-like changes of articular cartilage and loss of the chondrocyte phenotype[10,12]. Moreover, Wnt3a is not the only Wnt ligand which expression is increased in OA chondrocytes[2] and it can also activate non-canonical signaling pathways[3]. This indicates that a delicate balance in Wnt signaling is needed to keep chondrocyte phenotypic stability.

Further, heparan sulfate proteoglycans in the extracellular matrix (ECM) seem to modulate Wnt signaling by binding Wnt ligands and facilitating ligand–receptor interactions[13,14], thereby regulating cell sensitivity. Hence, several factors affect Wnt signal transduction, isolated chondrocytes might respond differently to external stimuli than they would respond *in vivo*.

Blockade of canonical Wnt signaling by SAH-Bcl9 and StAx-35R inhibited chondrocyte phenotypic shift in preserved cartilage resulting in increased SOX9 and ACAN, and decreased COL10A1 gene expression [Fig. 2(C)]. In OA lesion cartilage the Wnt inhibitors had no effect. We hypothesize that OA lesion cartilage does not contain enough viable chondrocytes to be targeted by the inhibitors and to revert the already made changes to the chondrocyte phenotype, as apoptosis of chondrocytes is increasing with OA progression[15].

The findings of this study give rise to a potential new therapeutic approach using peptidomimetic canonical Wnt inhibitors in treatment of early OA.

Author contributions

AH performed all experiments, analyzed the data and wrote the manuscript; CHL provided human OA cartilage samples and was involved in the discussions, K.B. provided human cartilage samples from the morgue; AG, LD and TG synthesized stapled peptide inhibitors; MB participated in processing human samples; TG and TP were involved in data analysis and discussion; and JB participated in data analysis, directed the project and wrote the manuscript.

Conflict of interest

All authors have no conflict of interest.

Role of funding source

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Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.joca.2018.02.908.

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